Immunoassays for Food Analysis

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1 INTRODUCTION

The U.S. Environmental Protection Agency and other federal and state regulators are interested in the presence and bioavailability of pesticide residues that remain on agricultural products after application. Tolerances for raw agricultural products have been established for residues of chlorpyrifos, an organophosphorous pesticide that is widely used on food commodities. Rapid screening methods are needed to determine chlorpyrifos (O,O-diethyl O-[3,5,6-trichloro-2-pyridyl]phosphorothioate) residues for nonoccupational and dietary exposure studies. Ideally, these methods should be sensitive, easy-to-perform, and provide data of known quality in a cost effective format. Immunochemical methods such as the enzyme-linked immunosorbent assay (ELISA) have been extensively used to determine pesticide residues in a variety of matrices. Previously, an ELISA was developed for determining foliar dislodgeable residues of chlorpyrifos in leaf washes from sprayed vegetation as a screening method to assess exposure (1). Furthermore, ELISA methods are currently being used to determine chlorpyrifos residues in foods, particularly those frequently consumed by infants and children (2).

Detection of chlorpyrifos residues in foods is commonly performed by gas chromatography; however, the sample cleanup required makes the procedure laborious and time consuming. As an alternative to gas chromatography, a fast and efficient extraction technique for chlorpyrifos from various baby foods has been developed. The method utilizes a sonic methanol extraction, followed by dilution and detection with either a magnetic particle enzyme linked immunosorbent assay (ELISA) from Strategic Diagnostics Incorporated or a plate ELISA. A similar ELISA method using homogenization to recover chlorpyrifos residues from produce has been reported (3), providing initial background for this work.

Within various regulating agencies, research and development studies are conducted to define appropriate analytical methods to enforce comprehensive risk-based standards (4). The EPA Office of Research and Development plans many of its projects around its risk paradigm which addresses hazard identification, quantitative exposure and dose assessment, and dose response. Effective risk assessments rely on analytical methods for the characterization and measurement of pesticides in environmental matrices. Since immunoassay data can provide the information necessary for risk characterization and management, immunoassay methods which support human exposure assessment studies are being explored.

2 TOLERANCES

The magnetic particle ELISA detected chlorpyrifos in the limited concentration range of 0 ppb - 3 ppb. In contrast, the plate assay offered more sensitivity because it detected chlorpyrifos within the broader concentration range of 0 ppb - 200 ppb. Despite these limitations, the detection levels of both ELISAs were well below the tolerance levels which are listed in the table below.

TABLE 1. CHLORPYRIFOS TOLERANCE LEVELS FOR FOODS COMMONLY FOUND IN THE DIETS OF INFANTS AND CHILDREN

Food	EPA Tolerance Level, ppm
Milk, solids	0.01
Applesauce	1.5
Oranges, fresh peeled fruit	1.0
Peaches, fresh	0.05
Pears, fresh	0.05
Carrots	NT
Milk, fat	0.25
Rice, milled	NT
Bananas, fresh	0.01
Grapes, fresh	1.0
Sweet peas, fresh	0.05
Beans, green	0.05
Oats	NT
Spinach, canned	NT
Tomatoes, fresh	1.0
Chicken products and byproducts	0.05

NT - No Tolerance Set (5)

Bolded foods are included on the USDA's list of 18 foods most consumed by nursing and nonnursing infants (6).

Commodities which are underlined were ingredients in the baby food tested: Banana Applesauce Dessert, Carrots, Peas, Creamed Spinach, and Chicken Noodle Dinner.

3 EXTRACTION PROCEDURE

FOOD SAMPLE PREPARATION FOR CHLORPYRIFOS 96-WELL INDIRECT IMMUNOASSAY

Spike 10 grams of food with chlorpyrifos spike
⇓
Add 20 mL of solvent and process with a Polytron homogenizer for three minutes
or
Add 20 mL of solvent, vortex, and sonicate for 30 minutes
⇓
Centrifuge for 5 minutes at 2000 rpm
⇓
Transfer supernatant to separate tube for storage
⇓
Dilute 1:10 with PBST buffer and analyze by ELISA
or
Concentrate samples by evaporation or Sep-Pak C_{18} Columns and dilute 1:10 prior to ELISA analysis
⇓
Solvent concentration is adjusted appropriately in the ten point standard curve
⇓
Follow ELISA protocol as described in frame 4

SONIC EXTRACTION OF CHLORPYRIFOS

Spike 10 grams of food with chlorpyrifos spike
⇓
Add 20 mL of solvent and vortex
⇓
Sonicate for 30 minutes
⇓
Centrifuge for 5 minutes at 2000 rpm
⇓
Transfer supernatant to separate tube for storage
⇓
Under N_2 gas, evaporate 100 µL of extract to near dryness
⇓
Reconstitute with 25 µL of MeOH
⇓
Transfer 12.5 µL into 237.5 µL of buffer and pipette 12.5 µL of MeOH into 237.5 µL of standard
⇓
Follow instructions per commercial* magnetic particle ELISA kit

**Commercial Assay produced by Strategic Diagnostics Inc. (Newark, Del.)*

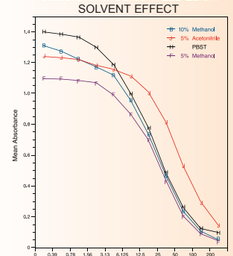
4 CHLORPYRIFOS ELISA PROTOCOL

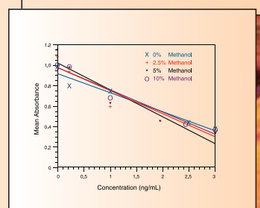
1. Passively adsorb 200 µL of a 125 ng/mL OVA-1 antigen to microtiter wells by incubation at 4° C overnight.
2. Wash plates three times with phosphate buffered saline containing Tween 20 (PBST). Seal all unused plates with acetate film, store for future use at 4° C.
3. Prepare a standard curve in PBST by serially diluting in a 1:2 ratio from 200 ng/mL to 0.198 ng/mL.
4. Add 100 µL of standards, sample, and blanks to appropriate well.
5. Add 100 µL of a 1:4000x dilution of chlorpyrifos monoclonal antibody in PBST. Substitute PBST for antibody in non-specific blank.
6. Cover plate and shake on an orbital shaker for two hours.
7. Wash plate three times with PBST, rotating plate twice in the process. Tap dry.
8. Add 200 µL of goat anti-mouse IgG, conjugated to alkaline phosphatase at a 1:1000x dilution.
9. Cover plate again and shake for an additional two hours.
10. Wash plate and add 200 µL of 1 mg/mL p-nitrophenyl phosphate in diethanolamine substrate. In 30 minutes, take an endpoint reading at 405 nM.
11. Analyze data with a four-parameter standard curve fit.

5 SOLVENT/MATRIX EFFECTS

A streamlined extraction procedure was developed to eliminate as many experimental steps as possible. Ideally, analysis would be as simple as adding an aliquot of methanolic extract to buffer and then proceeding with the ELISA. Direct addition, however, was problematic due to solvent interference with the immunoreactions. The graphs below indicate how various percentages of MeOH affected both ELISAs.

CHLORPYRIFOS 96-WELL ELISA SOLVENT EFFECT





In addition to the solvent effects, flavenoids and food preservatives can also interfere with assay results (7). Therefore the amount of food extract used was kept to a minimum. Table 6 demonstrates how the absorbance readings were impacted by the food matrices.

TABLE 6. MATRIX EFFECT ON IMMUNOASSAY SIGNAL

SAMPLE	ABSORBANCE
PBST Buffer + 10% MeOH	0.979
PBST Buffer Spiked at 5 ng/gm + 10% MeOH	0.737
Peas, 0 ng/gm chlorpyrifos	0.921
Peas, Spiked at 5 ng/gm chlorpyrifos	0.774
Carrots, 0 ng/gm chlorpyrifos	0.893
Carrots, Spiked at 5 ng/gm chlorpyrifos	0.832
Spinach, 0 ng/gm chlorpyrifos	0.866
Spinach, Spiked at 5 ng/gm chlorpyrifos	0.763

6 RECOVERIES

Ten gram food samples were spiked with various amounts of chlorpyrifos. Using the previously stated extraction protocol, chlorpyrifos extracts were analyzed with the commercial assay. Below, the data in table 2 indicated recoveries from a 200 ng/gm spike.

TABLE 2. RECOVERY FOR SONICATED, METHANOLIC EXTRACTS, 200 ng/gm SPIKE

FOOD	SAMPLE SIZE	AVERAGE % RECOVERY
Chicken Noodle Dinner	n = 11	93%
Carrots, Broccoli & Cheese	n = 4	94%
Banana Applesauce Dessert	n = 4	108%
Creamed Spinach	n = 4	96%
Carrots	n = 7	84%
Green Beans	n = 6	80%

In order for the food extract to contain detectable levels of chlorpyrifos at lower spiking levels, the samples were concentrated with evaporation before screening with the commercial assay. The results of the reduced spiking featured in table 3, compared the sonicated methanolic extracts from the same supernatant in both the commercial and plate assays.

TABLE 3. RECOVERY COMPARISON OF ELISA METHODS FOR SONICATED, METHANOLIC EXTRACTS, 20 ng/gm SPIKE

FOOD	SAMPLE SIZE	COMMERCIAL ELISA AVE. % RECOVERY	PLATE ELISA AVE. % RECOVERY
Creamed Spinach	n = 5	96%	106%
Carrots	n = 5	85%	82%
Peas	n = 5	85%	89%
Chicken Noodle Dinner	n = 5	84%	86%
Banana Applesauce Dessert	n = 5	89%	98%

TABLE 4. RECOVERY FOR HOMOGENIZED, METHANOLIC EXTRACT, 5 ng/gm SPIKE

FOOD	SAMPLE SIZE	AVERAGE % RECOVERY
Chicken Noodle Dinner	n = 9	88%
Banana Applesauce Dessert	n = 9	98%
Creamed Spinach	n = 9	89%
Carrots	n = 9	100%
Peas	n = 9	79%

With the plate ELISA, food was spiked at a lower level because the assay could withstand up to 10% methanol. For samples spiked at or below 5 ng/gm of chlorpyrifos, the extract was concentrated by either Sep-Pak C_{18} columns or evaporation under N_2 gas prior to analysis. With both procedures, 10 mL of extract was reduced to one mL of sample for quantitation at the one ng/gm level.

7 SEP-PAK CLEAN-UP

The initial sample prep protocol used in this food study was adapted from the Rodney Bushway, et al. study of chlorpyrifos in fruits and vegetables. According to Bushway's procedure (3), a 10-mL aliquot of methanolic food extract was diluted in 90 mL of HPLC-grade water. This 100-mL solution was then passed through an activated C_{18} Sep-Pak. The Sep-Pak was then dried under a light vacuum for 15 minutes, followed by a one mL elution with acetonitrile. For both the commercial and plate ELISA screens, sample loss occurred during this clean-up procedure with percent recoveries of chlorpyrifos ranging from 40 to 65%. In an attempt to increase the concentration of chlorpyrifos in the eluate, a larger sample load of 10 mL of methanolic extract was run through an activated column. Following the drying step, the column was again eluted with one mL of acetonitrile. The initial food extract was spiked at a level of 10 ng/gm, which is equivalent to 10 ng/mL of chlorpyrifos, therefore theoretically the Sep-Pak should have captured 100 ng. Hence, the chlorpyrifos concentration in the eluate (one mL of acetonitrile) should have been 100 ng/mL. By diluting the concentrated sample 1:10 with PBST, the plate ELISA analyzed for approximately 1 ng/gm chlorpyrifos. The table below gives the results of the modified procedure.

TABLE 5. CHLORPYRIFOS RECOVERY FROM A C_{18} SEP-PAK, SPIKED AT 1 ng/gm

FOOD	SAMPLE SIZE	AVERAGE % RECOVERY
Chicken Noodle Dinner	n = 5	93%
Spinach	n = 5	87%
Peas	n = 5	90%

8 SUMMARY

Chlorpyrifos was efficiently extracted from baby food in a procedure utilizing methanol extraction with sonication. Optimal conditions were experimentally determined to be 5-10% methanol in buffer with a 30 minutes sonication. The commercial assay tolerated a sample with a concentration of only 5% methanol, while the plate ELISA successfully recovered chlorpyrifos from a sample containing 10% methanol. At high spiking levels, 200 and 20 ng/gm, simple dilution was the sample preparation step necessary for chlorpyrifos detection. At a lower spiking level, a concentration step employing either C_{18} Sep-Paks or evaporation under N_2 gas was added before the ELISA procedure. The Sep-Pak procedure was optimized to reduce the number of experimental steps and to produce an eluate suitable for analysis. Given the high recoveries and the excellent detection capabilities of immunochemical methods, both the sample prep procedures and the assays described here will provide excellent analytical tools for future risk assessments.

9 REFERENCES

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(6) National Research Council, *Pesticides in the Diets of Infants and Children,* National Academy Press: Washington DC, 1993

(7) Manufacturer's instructions accompanying Food Prep Kit, Strategic Diagnostics Inc. (Newark, Del.)

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